Circadian clock gene *LATE ELONGATED HYPOCOTYL* directly regulates the timing of floral scent emission in *Petunia*

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Flowers present a complex display of signals to attract pollinators, including the emission of floral volatiles. Volatile emission is highly regulated, and many species restrict emissions to specific times of the day. This rhythmic emission of scent is regulated by the circadian clock; however, the mechanisms have remained unknown. In *Petunia hybrida*, volatile emissions are dominated by products of the floral volatile benzenoid/phenylpropanoid (FVBP) metabolic pathway. Here we demonstrate that the circadian clock gene *P. hybrida LATE ELONGATED HYPOCOTYL* (*LHY*; *PhLHY*) regulates the daily expression patterns of the FVBP pathway genes and floral volatile production. *PhLHY* expression peaks in the morning, antiphasic to the expression of *P. hybrida GIGANTEA* (*PhGI*), the master scent regulator *ODORANT1* (*ODO1*), and many other evening-expressed FVBP genes. Overexpression phenotypes of *PhLHY* in *Arabidopsis* caused an arrhythmic clock phenotype, which resembles those of *LHY* overexpressors. In *Petunia*, constitutive expression of *PhLHY* depressed the expression levels of *PhGI*, *ODO1*, evening-expressed FVBP pathway genes, and FVBP emission in flowers. Additionally, in the *Petunia* lines in which *PhLHY* expression was reduced, the timing of peak expression of *PhGI*, *ODO1*, and the FVBP pathway genes advanced to the morning. Moreover, PhLHY protein binds to *cis*-regulatory elements called evening elements that exist in promoters of *ODO1* and other FVBP genes. Thus, our results imply that PhLHY directly sets the timing of floral volatile emission by restricting the expression of *ODO1* and other FVBP genes to the evening in *Petunia*.

circadian rhythm | floral volatile | benzenoids | *Petunia hybrida* | LHY

Plant development and physiology are extensively influenced by the circadian clock (1). The precise timing of a single plant behavioral output often requires a suite of internal mechanisms to occur in coincidence or in quick succession before the behavior taking place. Transcriptome analysis revealed that the circadian clock controls transcription of one third of genes in *Arabidopsis* (2). In this way, the clock can exert a holistic effect on a complex mechanism at a precise moment in time. The effectiveness of the clock's ability to coordinate complex behaviors has been used by many aspects of plant physiology, such as photosynthesis, stem and leaf growth, and flowering (3, 4).

The precise timing of sexual reproductive events is critical, as plants are sessile and individuals are often spread over large distances. In addition to regulating the timing of flower formation, when they have opened, many flowers emit floral scents to lure pollinators. Attractive floral volatiles are often emitted in a rhythmic fashion, with peaks of emission coinciding with the primary pollinator's period of activity (5). Although studies have shown that rhythmic emission of scent requires the influence of a circadian clock (6–8), no study of which we are aware has shown a mechanistic link between clock function and floral volatile production.

Research on floral volatile synthesis has often used the common garden petunia, *Petunia hybrida* cv. Mitchell, which exhibits white flowers that peak in scent emission in the middle of night (9). Floral scent in *Petunia* is dominated by volatile benzenoid/phenylpropanoids (FVBP), a group of organic compounds originally derived from phenylalanine (5). FVBP emission relies on the availability of precursor compounds that flow from enzymatic reactions in the shikimate pathway and later throughout the FVBP metabolic pathways (Fig. 1*A*). *ODORANT1* (*ODO1*), a transcriptional activator gene of FVBP gene expression, exhibits a daily oscillatory expression with an evening peak (10). By binding to the promoters of several key enzymes, such as 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), ODO1 facilitates the introduction of precursor molecules to the FVBP synthesis pathway, suggesting that ODO1 is a master regulator of FVBP emissions in *Petunia*. Two transcription factors, EMISSION OF BENZENOIDS I (EOB I) and EOBII, up-regulate the expression of *ODO1* and other FVBP-related genes (11, 12).

Analysis of the *ODO1* promoter (12), as well as the promoters of several other regulatory genes in the FVBP pathway, revealed the presence of specific *cis*-elements referred to as evening elements (EEs). The EE is a binding site for two similar circadian-clock transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and a related factor, REVEILLE 8, in *Arabidopsis* (13). CCA1 and LHY, which are highly expressed during the morning, function as repressors for evening expressed genes (14, 15) and activators for morning expressed genes (16). Thus, we hypothesized that the *Petunia* ortholog of *CCA1*/*LHY*, through the repression of *ODO1* and several

Significance

Flowering plants attract pollinators in part by emitting volatile scents from their petals. This emission of scent is highly regulated, and is often restricted to a specific portion of the day. Although the biochemical pathways of scent production are well characterized, little is known of their transcriptional regulation. Here we describe a direct molecular link between the circadian clock and floral volatile emissions. We find that a clock transcription factor regulates the timing of multiple genes involved in the production of floral volatiles in *Petunia*. This work provides key insights into the complex yet relatively unexplored transcriptional regulation of scent production, and also sheds light on how the circadian clock can regulate the timing of large metabolic pathways.

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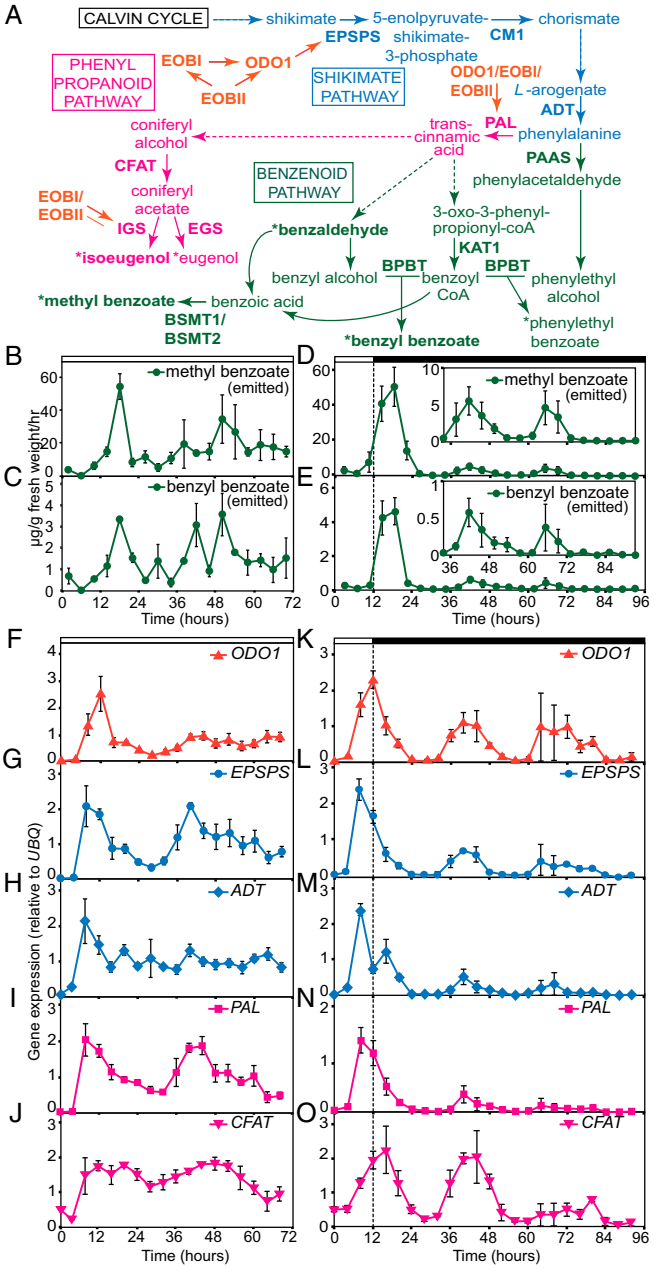

Fig. 1. The floral volatile emission and expression profiles of the genes in the FVBP pathway. (*A*) An overview of selected parts of the FVBP pathway. Different colors indicate steps and products that are categorized in shikimate, benzenoid, and phenyl propanoid pathways. Arrows with dashed lines in the pathway are representative of multiple steps between products. Volatile products are presented with asterisks. We analyzed expression patterns of enzyme genes and products in bold. Transcription factors are in orange, and enzymes shown are EPSPS, CM1, ADT, phenylacetaldehyde synthase (PAAS), BPBT, KAT1, S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (BSMT) 1, BSMT2, PAL, CFAT, IGS, and eugenol synthase 1 (EGS). (*B–E*) Volatile emission data of methyl benzoate (*B* and *D*) and benzyl benzoate (*C* and *E*) in *Petunia* in continuous light (*B* and *C*) and dark (*D* and *E*). (*Insets*, *D* and *E*) Graphs with enlarged *y*-axes showing the same 32–96 time point results. (*F–O*) Gene expression patterns of transcription factors and enzymes associated with the FVBP pathway in *Petunia* in continuous light (*F–J*) and dark (*K–O*). The line and symbol color of the graphs corresponds to its placement within the FVBP pathway shown in A. Values are relative to *UBIQUITIN* (*UBQ*), and normalized by the average expression values of hours 0–12. Results represent means ± SEM from three biological replicates. White and black bars at the top indicate periods of light and dark, respectively.

other regulatory genes, might restrict the emission of floral volatiles to the evening. Increasing levels of CCA1/LHY in the morning represses the expression of genes necessary for volatile synthesis, and as the levels of CCA1/LHY decrease in the evening, it facilitates the induction of *ODO1* and downstream FVBP enzyme gene expression. Here we report that the circadian clock regulates scent emission timing through the function of *LHY* ortholog in *Petunia*.

Results

The Circadian Clock Regulates Volatile Emission and the Expression of Enzyme Genes in the FVBP Pathway in the Dark. Previous work has shown that daily light/dark transitions are the predominant cues mediating temporal control of the major floral volatile, methyl benzoate, in *Petunia* (17). However, emission of the terpenoid compound β-ionone and the expression of its synthase enzyme showed circadian oscillations, particularly in the dark (8), indicating that the influence of the circadian clock on scent emission can be conditional. To more comprehensively assess the involvement of the circadian clock in scent emission, we measured the emission of four major floral volatiles (methyl benzoate, benzyl benzoate, benzaldehyde, and isoeugenol) generated through the FVBP pathway (Fig. 1*A*) under continuous light and dark conditions (Fig. 1 *B–E* and Fig. S1 *A–D*). In continuous light, volatile emissions ceased to oscillate, and moderate levels of emission were maintained for all four analyzed volatiles for 3 d (Fig. 1 *B* and *C* and Fig. S1 *A* and *B*). In continuous dark, volatile peak strength diminished rapidly (Fig. 1 *D* and *E* and Fig. S1 *C* and *D*); however, smaller peaks (of approximately one tenth of the size of the peaks that occurred in the light/dark conditions) were seen for all volatiles examined in the two following subjective nights, indicating the presence of circadian timing mechanisms in the dark.

Our current understanding of the transcriptional regulation of the FVBP pathway is limited to a few transcription factors: ODO1, EOBI, and EOBII (10–12). *ODO1* exhibits an evening peak (Fig. 1*K*), whereas *EOBI* and *EOBII* exhibit morning peaks (Fig. S1 *O* and *P*). To investigate the molecular link between the circadian clock and the FVBP pathway, we next analyzed the expression patterns of these three transcriptional regulators and 12 enzyme-encoding genes in the FVBP pathway under continuous light and dark conditions (Fig. 1 and Fig. S1). All FVBP synthesis genes examined showed strong daily oscillations in light/dark cycles (see the first 24-h pattern in Fig. 1 *K–O* and Fig. S1 *O–X*). In continuous light, the expression profiles of almost all genes did not sustain daily oscillatory patterns, and often the expression levels became higher than the trough levels observed in 24-h light/dark conditions (Fig. 1 *F–J* and Fig. S1 *E–N*). An exception was the benzoyl-CoA: benzyl alcohol/phenylethanol benzoyltransferase (*BPBT*) gene, which is expressed in a circadian fashion even in continuous light (Fig. S1*L*). Overall, their expression patterns resembled the emission profiles of the four volatiles examined under the same conditions (Fig. 1 *B* and *C* and Fig. S1 *A* and *B*). These results suggest that the circadian clock does not have much influence on the timing expression of the FVBP pathway genes as well as scent emission in continuous light in *P. hybrida* flowers.

Conversely, in continuous dark, *ODO1*, direct target genes of ODO1 [*EPSPS* and phenylalanine ammonia-lyase 1 (*PAL*) genes (10)], and several other enzyme-coding genes in the shikimate, benzenoid, and phenylpropanoid pathways [e.g., arogenate dehydratase 1, coniferyl alcohol acyltransferase (*CFAT*), and 3-ketoacyl-CoA thiolase 1 (*KAT1*) genes] displayed dampened but oscillatory mRNA expression patterns for 3 d (Fig. 1 *K–O* and Fig. S1 *O–X*), indicating the contribution of the circadian clock to the expression timing of these genes. *EOBI* and *EOBII* did not show any rhythmic expression patterns under these conditions, suggesting that daily rhythmic expression of *EOBI* and *EOBII* could largely be regulated by light/dark transitions. Also, this indicates that the daily rhythmic expression of *EOBI* and *EOBII* does not control the daily *ODO1* expression, even

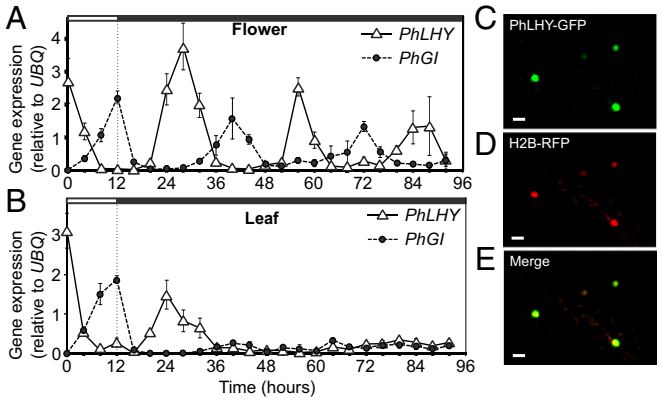

Fig. 2. Circadian expression pattern of *PhLHY* and intracellular localization of PhLHY protein. (*A* and *B*) Expression profiles of *PhLHY* and *PhGI* in continuous dark over 92 h in *Petunia* petals (*A*) and leaves (*B*). Results represent means ± SEM from three biological replicates. (*C*–*E*) PhLHY-GFP is a nuclear localized protein. PhLHY-GFP protein (*C*) and H2B-RFP protein (reference for nuclei) (*D*) were expressed in epidermal cells of *Petunia* petals. (*E*) Merged image of *C* and *D*. (Scale bar: 10 μm.)

though EOBs are direct activators of *ODO1* (11, 12). These results indicate that the circadian clock may contribute in the timing regulation of these genes in the dark in *Petunia* flowers. In addition, the present work and previous findings led us to hypothesize that the clock's regulation of volatile production may be modulated by light conditions. Another interesting finding is that *ODO1*, which directly up-regulates *EPSPS* and *PAL* expression (18), is expressed at the same time as *EPSPS* and *PAL*, especially in the dark (Fig. 1 *K*, *L*, and *N*). This suggests that additional factors are responsible for controlling the timing of the expression of these genes. We found that the *EPSPS* promoter possesses the EE *cis*-element (see Fig. 5*B*). As the *ODO1* (12) and the *EPSPS* promoters contain EEs, we hypothesized that CCA1/LHY homologs in *Petunia* may be negative factors that set the expression timing of these genes.

Identification of *LHY* Homolog in *Petunia*. To identify the *CCA1*/*LHY* homologous gene in *Petunia*, we first identified partial sequences that showed homology to *Arabidopsis CCA1*/*LHY* from publically available EST databases. After cloning the entire coding region of the cDNA based on the sequences of the EST clones, the entire sequence showed a higher homology to *LHY* than *CCA1*; therefore, we named it *P. hybrida LHY* (*PhLHY*). By using deduced amino acid sequences, Bayesian posterior probability and maximum-likelihood phylogenetic analyses placed PhLHY nested within the clade of core eudicots—clustered with *Nicotiana attenuata* LHY (NaLHY) and *Solanum lycopersicum* (*SlLHY*) (Fig. S2*A*). In addition, quantitative PCR (qPCR) analysis of *PhLHY* expression in *Petunia* leaves and flowers revealed peak expression at dawn under light/dark conditions (Fig. 2 *A* and *B* and Fig. S3 *A* and *B*), which is similar to *Arabidopsis CCA1*/*LHY* expression patterns (14, 15). To monitor the status of the *Petunia* circadian clock, the expression patterns of the *Petunia* homolog of *GIGANTEA* (*PhGI*), a clock gene directly regulated by CCA1 (19–21), was also analyzed (Fig. S2*C*). Similar to the *Arabidopsis* counterpart, *PhGI* expression peaks in the evening, demonstrating an antiphasic pattern to *PhLHY* expression. Interestingly, circadian oscillation of *PhLHY* and *PhGI* expression showed distinct patterns in tissue- and light-dependent manners. In *Petunia* flowers kept in continuous dark, robust oscillation of *PhLHY* and *PhGI* was observed for 3 d, whereas the circadian oscillation of *PhLHY* and *PhGI* in leaves ceased in the first day in the dark (Fig. 2 *A* and *B*). In continuous light, the amplitude of *PhLHY* expression levels was severely reduced in flowers and leaves, whereas the expression levels of *PhGI* remained similar to that in the light/dark cycle (Fig. S3 *A* and *B*). In addition, *PhGI* expression levels showed circadian oscillation in leaves, but not in flowers under continuous light conditions. These results indicate the presence of tissue-specific clocks between flowers and leaves in *Petunia*. Although the circadian oscillation patterns of *PhLHY* vary depending on tissues and light conditions, the daily expression patterns of *PhLHY* resemble those of *LHY*/*CCA1*.

Next we examined the intracellular localization of PhLHY protein, as its homologs CCA1 and LHY are nuclear-localized proteins (22, 23). In comparing the intracellular localization patterns of the PhLHY-GFP protein with that of RFP-tagged histone 2B (H2B), PhLHY-GFP localized in the nuclei of *Petunia* flower and leaf cells (Fig. 2 *C*–*E* and Fig. S3 *C*–*E*), indicating that PhLHY is a nuclear protein. Based on the sequence similarity, and the temporal and intracellular expression patterns, *PhLHY* is likely an orthologous gene of *Arabidopsis LHY*.

PhLHY Maintains Functional Relevance in *Arabidopsis*. To assess the function of *PhLHY*, we first tested whether it maintains a similar role with *Arabidopsis LHY*. *PhLHY* was overexpressed in *Arabidopsis* by using the cauliflower mosaic virus (CaMV) *35S* promoter (Fig. 3*A*). *PhLHY*-overexpressing lines (*35S*:*PhLHY*) displayed distinct developmental phenotypes typically observed in *Arabidopsis CCA1*/*LHY* overexpressors: a significantly delayed flowering time, and a longer hypocotyl length in comparison with WT plants (Col-0; Fig. 3 *B* and *C*) (14, 24). The *35S*:*PhLHY* lines also exhibited a similar clock arrhythmia phenotype to the *CCA1*/*LHY* overexpressors under continuous light conditions (14, 24), as measured by a *CCA1*:luciferase (*LUC*) reporter (Fig. 3*D*). In addition, the *CCA1*:*LUC* expression in *35S*:*PhLHY* barely responded to the dark-to-light transitions in the morning (Fig. 3*E*), indicating that the oscillation of circadian clock genes was severely attenuated even under light/dark conditions. Last, we analyzed the expression profiles of the circadian clock genes *LHY*, *CCA1*, *PSEUDO RESPONSE REGULATOR 9* (*PRR9*), *PRR7*, and *TIMING OF CAB EXPRESSION 1* (*TOC1*) in *35S*:*PhLHY* lines. The daily oscillation patterns of these genes became relatively constant in *35S*:*PhLHY* lines throughout the day (Fig. S4). Together, these results imply that *PhLHY* may possess a similar clock function to *CCA1*/*LHY* in *Arabidopsis*.

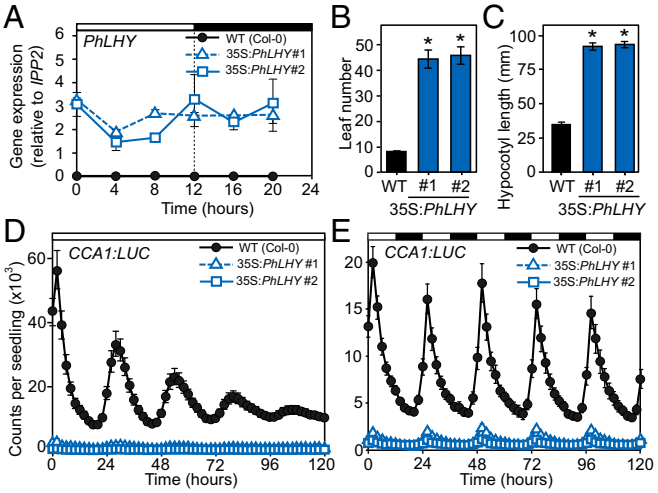

Fig. 3. *PhLHY* functionally resembles *CCA1*/*LHY* in *Arabidopsis*. (*A*) Expression of *PhLHY* in *35S*:*PhLHY* plants under light/dark conditions. (*B*) Flowering time of *35S*:*PhLHY* lines and WT plants is shown. (*Significant difference vs. WT at $P < 0.05$, Student *t* test; $n = 16$.) (*C*) Hypocotyl length of *35S*:*PhLHY* lines and WT plants (*$P < 0.05$; $n = 30$). (*D* and *E*) *CCA1*:*LUC* activity as measured by luminescence counts per seedling over 5 d of continuous light (*D*) and light/dark (*E*) conditions in a comparison between *35S*:*PhLHY* lines and WT. Results represent means ± SEM ($n = 16$).

PLANT BIOLOGY

Constitutive Expression of *PhLHY* Eliminates Floral Volatile Emission in *Petunia*. As *PhLHY* is likely an orthologous gene of *Arabidopsis LHY*, we next examined *PhLHY*'s influence on the daily production of floral scent in *Petunia*. Constitutive expression of *PhLHY* in *Petunia* (*35S*:*PhLHY* no. 37 line) altered expression patterns of two putative clock gene homologs [*PhGI* and *P. hybrida PSEUDO RESPONSE REGULATOR 5* (*PhPRR5*; Fig. S2*D*)] and 15 genes involved in the FVBP pathway in flowers (Fig. 4 *A*–*E* and Fig. S5 *A*–*M*). The expression levels of the genes [*PhGI*, *PhPRR5*, *ODO1*, *EPSPS*, chorismate mutase 1 (*CM1*), arogenate dehydratase 1 (*ADT*), and *PAL*], which peak at approximately 8–12 h from the morning in WT plants, became severely reduced and constitutive throughout the day. We further investigated that overexpression of *PhLHY* severely represses *ODO1* promoter activity in vivo. Transient coinfiltration of *ODO1* promoter-controlled firefly *LUC* gene reporter (*pODO1*:*LUC*) with *35S*:*PhLHY* (but not with *35S*:*GFP*) suppresses diurnal oscillation patterns of the *ODO1* promoter activities in multiple independent *Petunia* flowers (Fig. S6). Taken together, these expression patterns of evening-expressed genes in *35S*:*PhLHY* no. 37 and in the transient assay were similar to those in *CCA1*/*LHY* overexpressors in *Arabidopsis* (14, 24). Additionally, constitutive expression of *PhLHY* in *Petunia* resulted in a near-total decrease in floral volatile production and emission (Fig. 4 *P* and *Q*, and Fig. S5 *N* and *O*). These results indicate that the *PhLHY* expression pattern controls the expression patterns of other clock gene homologs and FVBP pathway genes in addition to the scent emission patterns in flowers.

Repression of *PhLHY* Leads to the Early Production of Scent. *Petunia* is well known for gene silencing resulting from attempts to express transgenes (25, 26). Our attempt to constitutively express *PhLHY* also resulted in transgenic plants that exhibited silencing of *PhLHY*. In the plants with reduced expression levels of *PhLHY*, the phases of peak expression of *PhGI* and *PhPRR5* as well as most of the FVBP pathway genes responsible for volatile precursor synthesis (*EOBI*, *EOBII*, *ODO1*, *EPSPS*, *CM1*, *ADT*, and *PAL*), shifted approximately 4–8 h toward the morning (Fig. 4 *F*–*O* and Fig. S5 *U*–*AG* and *AO*–*BA*). This phase-advance phenotype is also observed in *cca1 lhy* double mutants in *Arabidopsis* (15, 27). Interestingly, the volatile emission peaks in these plants also occurred at the end of the light period (approximately 8–12 h from the morning) instead of the middle of the night, although the total scent emission levels were slightly lowered in line 46 (Fig. 4 *R*–*U* and Fig. S5 *AH*–*HI*, *BB*, and *BC*). These results imply that PhLHY sets the expression timing of evening-expressed FVBP pathway genes.

PhLHY Binds to the EEs in *ODO1*, *EPSPS*, and Isoeugenal Synthase 1 Promoters. Our results indicated that PhLHY regulates the timing of FVBP pathway genes in *Petunia*. The *ODO1* promoter contains several EEs (12), and we found that the promoter sequences of the evening-expressing *EPSPS* and morning-expressing isoeugenol synthase 1 (*IGS*) also contain EEs. Thus, we hypothesized that PhLHY directly binds to these EEs to regulate the temporal expression of these genes. To analyze the direct binding of PhLHY to the EEs, we performed an EMSA of PhLHY (Fig. 5 *A* and *B*). The glutathione S-transferase (GST)-fused PhLHY protein specifically bound to the two EEs (EE1 and EE2) and the CCA1-binding site (CBS) (28) derived from the *ODO1* promoter, but not to the mutated EE (mEE1; Fig. 5*A*). In addition, PhLHY also bound to the EEs in *EPSPS* and *IGS* promoters (Fig. 5*B*). We also tested the functional interaction of PhLHY on the EEs and CBS in the *ODO1* promoter in vivo by using transient expression assay (Fig. 5*C*). We found that coinfiltration with *35S*:*PhLHY* specifically reduced luminescence from *pODO1*:*LUC* in flowers while having no effect on an activity of the *ODO1* promoter with its EE and CBS sites mutated (*pODO1*:*LUC* mEEs + mCBS; Fig. 5*D*), further confirming that PhLHY represses *ODO1* by directly binding to its promoter.

Discussion

Identification of the Circadian Clock Gene *PhLHY* in *Petunia*. Our investigation into the clock's regulation of scent emission implicated a *P. hybrida* ortholog of *CCA1*/*LHY*, which we named

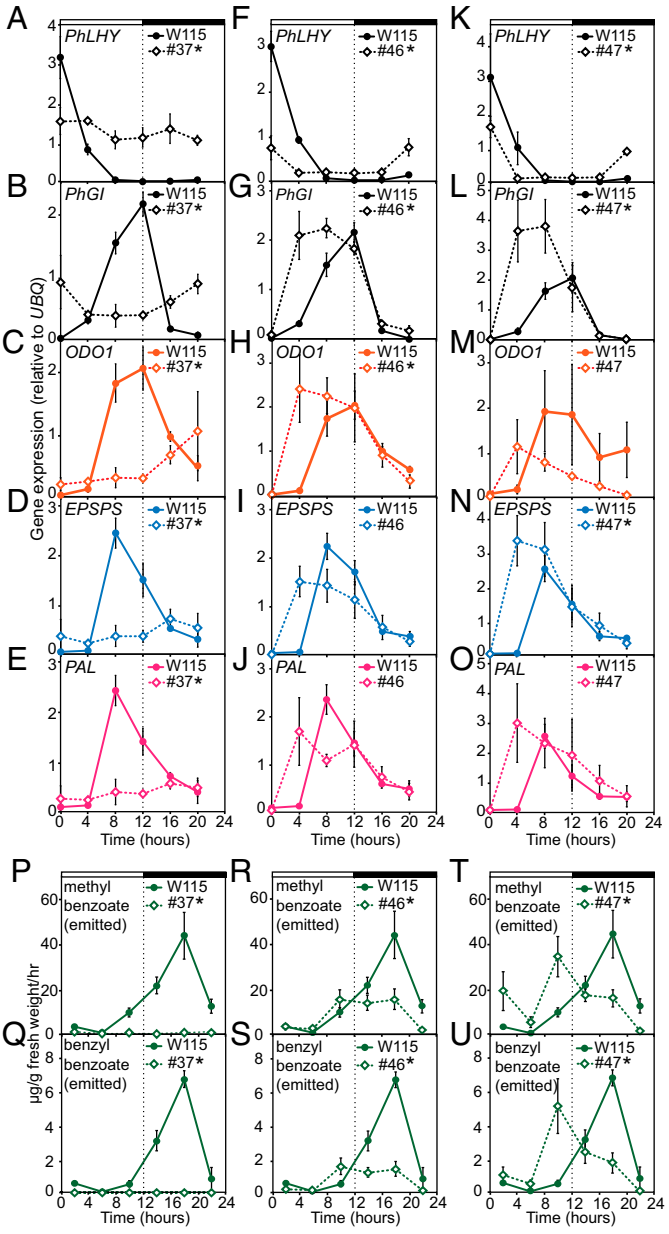

Fig. 4. *PhLHY* regulates daily timing of gene expression and volatile emission in the FVBP pathway. (*A*–*O*) Daily expression patterns of clock genes and genes encoding proteins in the FVBP pathway in the transgenic line (line 37) with constitutive *PhLHY* expression (*A*–*E*) and in the transgenic lines (lines 46 and 47) with altered *PhLHY* expression (*F*–*O*). Gene expression values were normalized by the average expression values of hours 0–12. (*P*–*U*) Daily scent emission patterns of methyl benzoate (*P*, *R*, and *T*) and benzyl benzoate (*Q*, *S*, and *U*) in transgenic line 37 (*P* and *Q*) and in transgenic lines 46 and 47 (*R*–*U*). Results represent means ± SEM from three biological replicates. The line color of the graphs corresponds to its placement within the FVBP pathway (Fig. 1*A*; $*P < 0.05$, daily expression and scent emission patterns of transgenic lines differ significantly from WT *Petunia*; two-way ANOVA).

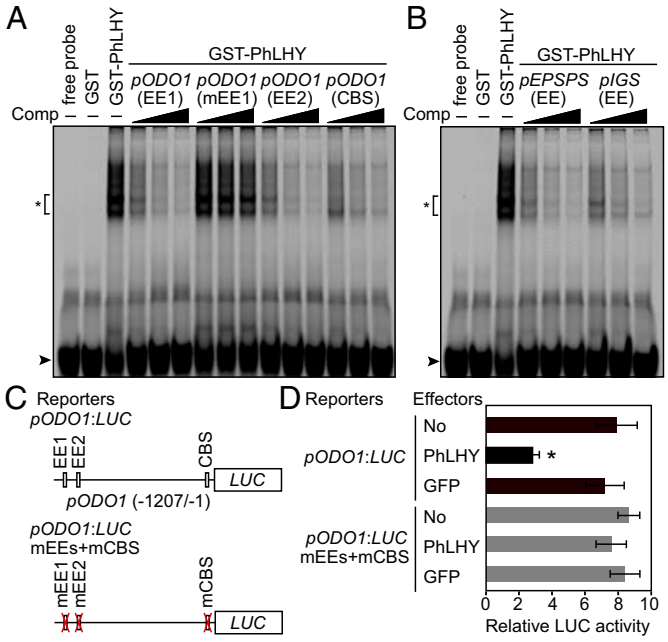

Fig. 5. PhLHY binds to EEs. (*A*) An EMSA shows direct interaction of GST-PhLHY with EEs in the *ODO1* promoter. The EE1 in the *ODO1* promoter (*pODO1*) was used as a labeled probe. Competition with different concentrations of unlabeled EE1, EE2, and CBS fragments and the mutated EE1 are shown along the top. (*B*) EMSA of GST-PhLHY with EEs in the *EPSPS* (*pEPSPS*) and *IGS* (*pIGS*) promoters. For *A* and *B*, GST served as a negative control. Asterisks and arrowheads indicate GST-PhLHY/DNA complexes and free probes, respectively. (*C*) Schematic of reporters used in transient assay. (*D*) The effect of PhLHY protein on the *ODO1* promoter activities. The activities of firefly LUC were normalized by the activities of *35S*:Renilla LUC. Results represent means ± SEM of nine independent samples (*$P < 0.01$ vs. no effector, Student *t* test).

PhLHY, as a mechanistic component. Our phylogenetic analysis placed PhLHY as the closest homolog of other Solanaceae LHY homologs (Fig. S2). The structure of this phylogenetic tree also resembled independent tree structures found in separate analyses (29, 30). qPCR analysis revealed rhythmic oscillation in *PhLHY* expression in *Petunia* floral tissues in continuous dark (Fig. 2). The daily expression patterns of *PhLHY* transcripts and the nuclear localization of the PhLHY protein (Fig. 2) provided additional support to the notion that *PhLHY* acts within *Petunia* in a manner consistent with its homologs (29, 30).

Arabidopsis PhLHY-overexpressing lines displayed several phenotypes similar to established clock-disrupting *CCA1*/*LHY*-overexpressing phenotypes. These lines showed delayed flowering times and elongated hypocotyls compared with WT plants (Fig. 3) (29, 30). In these lines, the disruption of the rhythmic expression of many circadian-clock genes was observed (Fig. 3 and Fig. S4). The disruption of these rhythms by *PhLHY* overexpression suggests that the function of *PhLHY* is similar to *LHY* and *CCA1*, in which overexpression caused general impairment of clock function (29, 30). As seen in numerous examples in animals and plants, clock components are often a part of a negative autoregulatory feedback loop in which the protein products of clock component genes suppress the expression of their own genes (29, 30). By comparing the *CCA1*:*LUC* luminescence and *CCA1* and *LHY* expression patterns of *Arabidopsis PhLHY* overexpressors and WT plants under continuous light and light/dark conditions, *PhLHY* overexpression depressed the rhythmic circadian oscillation of *LHY* and *CCA1* (Fig. 3 and Fig. S4). This same pattern of feedback regulation on *LHY* and *CCA1* was observed under conditions in which *LHY* and *CCA1* were overexpressed separately (29, 30), further supporting that the function of *PhLHY* resembles that of *LHY* and *CCA1*, and that *PhLHY* encodes a core clock component in *Petunia*.

PhLHY Regulates the Daily Timing of the FVBP Pathway. ODO1 is a key transcriptional regulator for many steps in the FVBP pathway (10). ODO1 regulates fragrance biosynthesis by administering the flux of precursor molecules through the shikimate pathway (5). Although *ODO1* exhibits rhythmic expression that peaks in the evening, its peak of expression closely mirrors that of the known ODO1 target genes (Fig. 1). This coincidence of expression suggests that *ODO1* is not wholly responsible for the precise timing of FVBP biosynthesis.

Under light/dark conditions, *PhLHY* morning expression is antiphasic to the evening expression of many FVBP-related genes including *ODO1*, *EPSPS*, and *PAL* (Fig. 4). Constitutive expression of *PhLHY* resulted in the abolishment of nearly all FVBP emission (Fig. 4 and Fig. S5) and suppressed most of the known genes responsible for the synthesis of FVBPs (Fig. 4 and Figs. S5 and S6). Under constitutive expression of *PhLHY*, *BPBT* and *BSMT* expression levels were increased (Fig. S5). As *ODO1* RNAi knockdown lines also showed increased levels of *BPBT* and *BSMT* expression (10), this phenotype might be induced by the low expression level of *ODO1*, which was caused by constitutive *PhLHY* expression. In *PhLHY*-suppressed lines, the peak expression of many FVBP genes examined shifted from late afternoon to earlier in the day (Fig. 4 and Fig. S5). This shift in peaks was seen most clearly in the precursor level of FVBP synthesis, with distinct shifts occurring in *EOBI*, *EOBII*, *ODO1*, *EPSPS*, *CM1*, *ADT*, and *PAL*. These data clearly suggest that PhLHY regulates the timing of FVBP emission by temporally controlling the expression profiles of enzyme-encoding genes that affect the synthesis of FVBP precursors. Furthermore, PhLHY directly bound to the EE and/or CBS *cis*-elements in the promoters of *ODO1*, *EPSPS*, and *IGS* (Fig. 5), indicating that PhLHY is likely setting the phase of *ODO1* and *EPSPS* expression by inhibition (and *IGS* through activation) during the early portion of the day. Based on the similarities of the expression patterns, our expression analyses also suggest that PhLHY may interact with the promoters of other FVBP genes (in perhaps both suppression and activation).

Our analysis of endogenous FVBP compound concentrations (Fig. S5) revealed that the temporal availability of endogenous compounds mirror the external emission levels of those same compounds. These findings support previous evidence that emission of floral volatiles is simply based on diffusion, and regulated primarily at the synthesis level (31, 32).

***Petunia* Likely Possesses a Tissue-Specific Clock that Regulates the FVBP Pathway.** Another interesting result was the apparent tissue specificity of the clock homolog expression in *Petunia*. *PhLHY* and *PhGI* show circadian rhythmic expression in flower tissue during continuous dark conditions, but not in continuous light. Correspondingly, a small but significant oscillation is observed in many FVBP genes also only during continuous dark. In leaf tissue, no circadian rhythm was present in continuous dark, but the expression profile of *PhGI* (but not that of *PhLHY*) shows a strong daily oscillation in continuous light. To regulate the robust oscillation of *PhGI* in leaves, clock components other than PhLHY may be involved. These results clearly indicate that *P. hybrida* plants likely possess circadian clocks that have different properties in flowers and leaves. Although tissue specificity of the circadian clock has been shown in *Arabidopsis* previously (33–35), current examples in other plant species are still limited (36). Of note is the fact that the common study organism for FVBP research, *P. hybrida*, is a hybrid of *Petunia axillaris* and *Petunia integrifolia*. *P. axillaris* shows robust oscillation of floral scent in continuous light conditions, but *P. integrifolia* does not (37). This indicates that the light- and tissue-specific clock phenotypes

observed in *P. hybrida* might be caused by mix of features of *P. axillaris* and *P. integrifolia* circadian clocks. This also suggests that there may be different characteristics in the molecular clocks even among *Petunia* species. Although robust circadian rhythms do not seem to persist in *P. hybrida*'s volatile release under continuous light conditions, the strong effect PhLHY has on FVBP synthesis under light/dark cycle conditions clearly indicates the importance of the clock to the timing of floral emissions in a real world in this species. Together with the light-dependent tissue specificity of rhythmic gene expression, it appears that light and the circadian clock work in concert to regulate floral volatile emissions in *Petunia*.

In summary, we describe what is, to our knowledge, the first molecular-level evidence that the clock component, PhLHY, plays an important role in controlling the timing of volatile synthesis and emission in *Petunia.* During an early part of the day, PhLHY represses the expression of many enzyme-encoding genes in the FVBP biosynthesis pathway, primarily in the upstream area of the pathway responsible for precursor availability. It is also possible that PhLHY is up-regulating certain morning-peaked FVBP genes, such as *IGS*. Further identification and analysis of FVBP regulatory components is required to provide a more precise level of control within the FVBP metabolic pathway. A fuller comprehension of the regulatory network surrounding the FVBP pathway will allow us to not only regulate the abundance of floral volatiles, but the period of emission as well. Such manipulation would allow us to create "designer crops" whose emissions could be adjusted to suit the temporal availability of local pollinators.

Materials and Methods

A full description of the materials and methods is provided in *SI Materials and Methods*. In brief, we created transgenic *Petunia* plants through tissue culture transformation by using *Agrobacterium tumefaciens*. Gene expression was measured through qPCR analysis, and all primer information for qPCR in this research is provided in Table S1. Volatile emissions were collected from live plants in a custom-designed apparatus (Fig. S7) and analyzed by GC/MS. Physical binding of PhLHY to EEs was confirmed by using EMSA and transient LUC assays.

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